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Hibernation, a state of natural tolerance to profound reduction in organ blood flow and oxygen delivery capacity

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1. Introduction – general features

Hibernation represents a seasonal physiological adaptation, which allows conservation of energy and down-regulation of cellular functions. Hibernating animals exhibit multiple biological alterations which contribute to their dramatic ability to tolerate the ischaemic conditions associated with reduced rates of respiration and blood flow while in the hibernating state. There is accumulating evidence that mammalian hibernation involves a controlled suppression of interactive physiological responses that preserves homeostatic balance. Mammalian hibernation is a regulated state of torpor with a profound suppression of energy requirements that has evolved in at least six mammalian orders as a strategy to cope with seasonal cold and shortages of food and water (Wang, 1988). A hibernation bout may last up to several weeks in some mammals and consists of entry into, maintenance of, and arousal from hibernation. Arousal is maintained for a few hours to a few days before the animal initiates another bout of hibernation.

During entrance into hibernation, metabolic rate drops to extremely low levels. In studies by Frerichs *et al.* (1995), glucose utilization was determined by the autoradiographic [^{14}C] deoxyglucose technique by direct chemical measurement (HPLC) of precursor and products in samples dissected from funnel-frozen brain. The calculated glucose metabolic rate (CMR_{Glc}) in ground squirrels in deep hibernation was only

Molecular Mechanisms of Metabolic Arrest: Life in Limbo, edited by K. B. Storey.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
2001	Book Chapter	

4. TITLE AND SUBTITLE

Hibernation, a state of natural tolerance to profound reduction in organ blood flow and oxygen delivery capacity

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Naval Medical Research Center
503 Robert Grant Ave.
Silver Spring, MD 20910

8. PERFORMING ORGANIZATION REPORT NUMBER

NMRC 01-026

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Bureau of Medicine and Surgery
(MED-02)
2300 E Street NW
Washington, DC 20372-5300

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Unlimited

13. SUPPLEMENTARY NOTES

Published in: Storey HB, ed. Molecular Mechanisms of Metabolic Arrest: Life in Limbo. Oxford: Scientific Publishers Ltd., pp. 23-42, 2001.

14. ABSTRACT

None

15. SUBJECT TERMS

Hibernation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	SAR	20	Aletha Holser
					19b. TELEPHONE NUMBER *(Include area code)* 301-319-9665

1–2% of the values in active animals. This study represented the first quantitative determination of CMR_{Glc} *in vivo* during hibernation and it demonstrated that metabolic balance was maintained despite severely reduced cerebral blood flows.

In addition to a decline in metabolic rate, the body temperature of hibernating animals falls to within several degrees of the ambient temperature; for example, ground squirrels hibernating at 1–2°C showed a core body temperature of 5°C (Frerichs *et al.*, 1994). Heart rate in hibernating ground squirrels can be in the range of 5–10 beats min^{-1} (normal 350–400 beats min^{-1}); respiratory rate has been noted to decrease to less than 1 min^{-1} (from a euthermic breathing rate above 40 min^{-1}). Blood pressure may drop from 130/80 mmHg to 90/30 mmHg and cardiac output may fall to 1/60 of the euthermic level (Wang, 1988). The EEG becomes isoelectric and the EKG remains normal during hibernation except for the bradycardia. The time courses of the decrease in body temperature and heart rate during entrance into hibernation differ as do the restorations toward normal during arousal; in both cases the rate of change in heart rate is greater than the rate of change in body temperature and occurs before the change in body temperature. Circulating levels of platelets, neutrophils, lymphocytes and monocytes fall to very low levels during hibernation within 1–2 hours of the entrance period and normalize during arousal within a comparable time period. In summary, the entrance into hibernation is characterized by dramatic reductions in body temperature, as well as metabolism, cardiac pulse and respiratory rate (Lyman and Chatfield, 1955; Frerichs *et al.*, 1994; Atanassov *et al.*, 1995). During bouts of hibernation, ground squirrel cerebral blood flow is reduced to ischaemic levels (Frerichs *et al.*, 1994; Atanassov *et al.*, 1995). Average cerebral blood flow, as determined by the [^{14}C] iodoantipyrine method (Sakurada *et al.*, 1978), was reduced in the brains of hibernating animals to 7 ± 2 ml 100 g^{-1} min^{-1} from 62 ± 18 ml 100 g^{-1} min^{-1} in controls. This severely reduced blood flow and oxygen availability would provoke brain damage in animals that do not normally hibernate (i.e., euthermic animals) but such drops in blood flow to 'ischaemic' levels are tolerated without damage in hibernating animals.

In hibernation, organs are able to survive long periods of time under very severe circumstances without any ultimate loss of function. The absence of neuropathological abnormalities as seen by light microscopy of cresyl violet-stained sections of brains obtained during hibernation and following arousal from hibernation indicates a tolerance to these ischaemic and hypothermic conditions. This state of tolerance is highly regulated by a plethora of alterations such as: 1. inactivation of mitochondrial function (Brustovetsky *et al.*, 1989; Martin *et al.*, 1999); 2. increased production of antioxidants (e.g., ascorbate) (Drew et al., 1999) and acute phase reactant proteins (e.g., alpha-1-antitrypsin) (Srere *et al.*, 1995; Takamatsu *et al.*, 1997); 3. increased expression of uncoupling proteins, which are inner mitochondrial membrane transporters that dissipate the proton gradient, releasing stored energy as heat, without coupling to other energy-consuming processes (Liu *et al.*, 1998); and 4. decreased metabolism through changes in body temperature (hypothermia) (Song *et al.*, 1995). These and other areas of study are involved in endeavours to analyse the regulation of hibernation as a natural state of tolerance to severely reduced blood flow and oxygen availability.

2. Modulation of enzyme activity in hibernation

The mechanisms that regulate metabolic depression in hibernation appear to involve a coordinated reduction of the activity state of key regulatory enzymes or proteins in

the cell (Storey and Storey, 1990). Based on assays of individual enzyme activities in liver extracts from hibernating jumping mice, depression of glycolysis was attributed to dephosphorylation of glycogen phosphorylase (in addition to a decrease in total glycogen phosphorylase protein), and phosphorylation of 6-phosphofructose-1-kinase and pyruvate kinase (Storey, 1987). The absolute rate of succinate-supported mitochondrial respiration is reduced by 50–70% in hibernating ground squirrels at all assay temperatures between 4°C and 37°C as compared to values in summer and winter euthermic animals (Pehowich and Wang, 1984). This indicates that inhibition of this enzyme in hibernation is not critically dependent on cold temperatures. In crude extracts of skeletal muscle from the hibernating jerboa (*Jaculus orientalis*), a small rodent of the Moroccan highlands, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) displays a decreased enzyme activity as a result of a change in the ratio of a catalytically inefficient isoform to a catalytically efficient isoform (Soukri *et al.*, 1995). In this species, GAPDH is regulated at a transcriptional level in muscle and at a post-translational level in liver (Soukri *et al.*, 1996).

3. Hibernation induction factors

There have been a number of reports of factors isolated from blood or extracted from tissues that produce hibernation or hibernation-like states when injected into animals. The variety of factors postulated as responsible for regulating the state of hibernation include proteins (Oeltgen *et al.*, 1978; Kondo and Kondo, 1992), hormones (Hermes *et al.*, 1993; Boswell *et al.*, 1994) and opioids (Cui *et al.*, 1993; Ziganshin *et al.*, 1994). Several groups have reported the isolation of a partially characterized factor from plasma of hibernating animals that induces hibernation in active ground squirrels exposed to cold in an environmental chamber during the summer (Dawe and Spurrier, 1969; Ruit *et al.*, 1987; Horton *et al.*, 1998); studies indicated that the active factor(s) were associated with the albumin fraction of plasma (Oeltgen *et al.*, 1978). The specificity and validity of the summer hibernation bioassay have been called into question and when proper controls were used, the putative 'hibernation induction trigger' fractions above did not give consistent results (Wang *et al.*, 1988). Extracts of brain and other tissues from hibernating ground squirrels (Swan and Schätte, 1977; Amorese *et al.*, 1982; Kramarova *et al.*, 1992; Ziganshin *et al.*, 1996) and from other hibernating (Sukhova, *et al.*, 1990) or aestivating (Swan *et al.*, 1968) animals have been observed to cause torpor, metabolic depression, reduced oxygen consumption and a drop in body temperature when injected into rats. Lyophilized serum albumin fractions containing putative 'hibernation induction trigger' extracted from the blood of hibernating woodchucks caused hypothermia and hypophagia when injected intracerebroventricularly into macaque monkeys (Myers *et al.*, 1981). Albumin from summer active woodchucks or bovine serum albumin in the identical range of doses had no effect. Several groups have reported that hibernation is influenced by serotonergic neurons in the median raphe nucleus (Spafford and Pengelley, 1971; Canguilhem *et al.*, 1986). The ability of extracts from hibernating but not active ground squirrels to cause hypometabolic and hypothermic effects in a homeotherm such as the rat further indicate the capacity of crude extracts to induce physiological changes (i.e., torpor) which resemble, albeit it to a lesser extent, the onset of hibernation (Karmanova, 1995). Despite considerable research by many laboratories and these reports of partially characterized hibernation induction factors, the detailed mechanisms by which hibernation is induced and maintained remain unclear.

4. Hibernation specific factors

4.1 *Plasma*

Recent experiments in our laboratory have attempted to identify proteins, which may be responsible for inducing a hypometabolic state, in the blood of hibernating animals. Previous investigations have identified and partially sequenced an 88 kDa protein called the hibernation-related factor (HRF) in plasma of hibernating woodchucks (Horton *et al.*, 1998) and prairie dogs (Bruce *et al.*, 1997) which is predominantly present during winter but down-regulated (in prairie dogs) or totally absent (in woodchucks) during the rest of the year. This protein is thought to be the active factor in plasma from hibernating woodchucks and may be involved in the capacity of the plasma to induce hibernation (Dawe and Spurrier, 1969), inhibit contraction of guinea pig ileum (Bruce *et al.*, 1992), and protect rabbit hearts from ischaemia-reperfusion injury (Bolling *et al.*, 1998). In our current experiments, pooled plasma samples from groups of hibernating or winter-active ground squirrels were chromatographically separated on Affi-Gel Blue (Bio-Rad) into adherent and non-adherent fractions, as described by Oeltgen *et al.* (1978). Affi-Gel Blue is a crosslinked agarose gel support with covalently coupled Cibacron Blue F3GA dye as the active ligand. The Affi-Gel Blue adherent fractions, which primarily consist of albumin, coagulation proteins, interferon, and enzymes, were analysed by 2-dimensional electrophoresis (isoelectric focusing (IEF) in tube gels followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on flat-bed gels). The gels were stained with coomasie blue, dried, and analysed by densitometric scanning (Molecular Dynamics; Sunnyvale, CA). These analyses indicated that the majority of proteins in the pooled sample of hibernating plasma were down-regulated; however, a few proteins were considerably (greater than twenty-fold) up-regulated (*Table 1*). The 2D gel experiments provide additional evidence for the existence of hibernation-specific differences in plasmas. The role of these proteins in metabolic- or temperature-specific alterations (or possibly other hibernation-related changes) is currently being investigated.

Table 1. Relative expression of plasma proteins[a]

Spot number	Active	Hibernating	H:A Ratio
1	1.0	25.0	25.0
2	32.6	4.8	0.1
3	73.7	1.0	<0.1
4	1.1	27.8	24.8
5	39.7	0.3	<0.1
6	74.3	12.9	0.2
7	450.5	10.5	<0.1
8	32.6	3.0	0.1
9	26.2	1.0	<0.1
10	36.2	1.0	<0.1
11	43.9	3.6	0.1

[a]Coomassie blue stained spots on 2 dimensional gels (isoelectrofocusing followed by SDS-polyacrylamide gel electrophoresis) were quantified by scanning on a densitometer and analysis by ImageQuaNT software (Molecular Dynamics, Inc, Sunnyvale, CA). Numbers represent the relative integrated pixel intensity excluding background.

4.2 *Plasma – effects on cell division*

During hibernation, the mitotic activity of cells and tissues has been shown to be dramatically decreased: mitoses are absent or are displayed as atypical forms. In tissues with typically high mitotic activity such as lymphoid tissue, DNA synthesis is suppressed; this inhibition of cell division is most notably manifest in the animal as an impaired immune function (Cooper *et al.*, 1992) and an inability to produce an antibody response during hibernation (Sidky and Auerbach, 1968). This biological adaptation lends itself as a convenient assay for bioactive factors in the plasma that might control cellular metabolism through inhibition of cell division. Previously, other investigators have noted that extracts from intestinal tissue of hibernating arctic ground squirrels induced body temperature depression and decreased oxygen consumption in mice (Karmanova, 1995); these extracts also inhibited proliferation of tissue-cultured cells. Plasma from hibernating woodchucks also inhibited growth of nutrient-starved cultured cells (Chien and Oeltgen, 1993).

The classical *in vitro* assay using freshly dissociated mouse spleen cells induced to proliferate by the mitogen concanavalin A (Con A) was utilized to investigate the presence of a growth-modulating factor in the plasma of hibernating 13-lined ground squirrels and woodchucks. Plasma samples and Con A were added to cultured BALB/cByJ mouse spleens cells and DNA synthesis was assessed 48 h later by measuring uptake of tritiated thymidine during a 4 h pulse. The presence of either hibernating or summer-active woodchuck or ground squirrel plasma depressed the tritiated thymidine uptake (*Figure 1*), however, the plasma from hibernating animals was significantly more inhibitory than the plasma from summer-active animals (*Figure 1*). These results confirm and extend the findings of Chien and Oeltgen (1993) in identifying a growth inhibitory activity in plasma of hibernating animals. This factor appears to be present in the plasma in both winter and summer, although in higher concentrations in winter, similar to the HRF activity in prairie dogs (Bruce *et al.*, 1997).

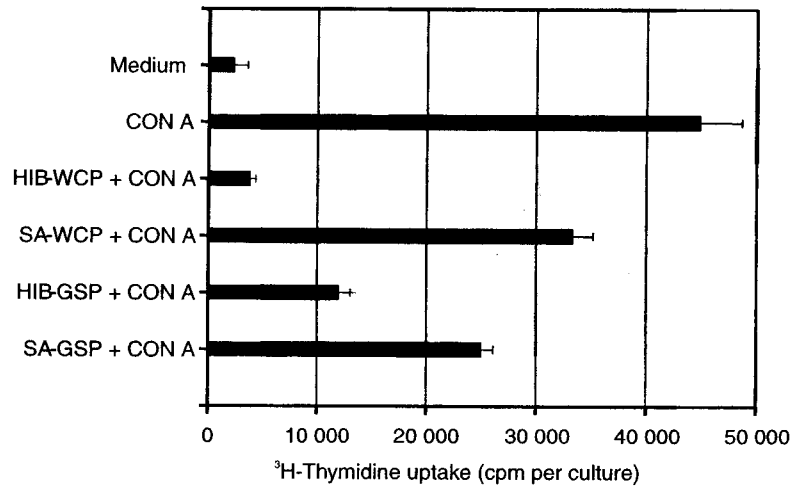

Figure 1. *Effects of 6% v/v plasma from hibernating (HIB) or summer-active (SA) 13-lined ground squirrels (GSP) or woodchucks (WCP) on Con A-induced proliferation of mouse spleen cells. Results represent the geometric mean CPM uptake of triplicate cultures +/– the standard error.*

4.3 *Plasma – effects on protein synthesis*

A second bioassay used for evaluating possible effects of plasma from hibernating animals in down-regulating the metabolism of cultured mammalian cells used the EL4 mouse lymphoma cell line in an assay to measure protein synthesis by uptake of ^{3}H-leucine. EL4 cells were cultured with various dilutions of 13-lined ground squirrel plasma from individual hibernating or winter-active squirrels. The cultures were pulsed with ^{3}H-leucine at their initiation, and replicate cultures were assayed for leucine uptake after 2 and 4 h of culture. Incubation with 6% plasma from hibernating animals significantly suppressed the level of EL4 protein synthesis (*Figure 2*). The degree of plasma-induced inhibition was dose-dependent; proportionately less inhibition was seen with plasma concentrations of 1.2 and 0.6% (data not shown). Similar findings were observed with hibernating woodchuck plasma and dog kidney epithelial cells (Chien and Oeltgen, 1993). The data suggest the presence of factors in the plasma of hibernating ground squirrels, which can regulate protein synthesis; the inhibition of protein synthesis may be a mechanism for down-regulation of cellular metabolism.

4.4 *Plasma – effects on cell adhesive interactions*

Circulating leukocytes decrease to about 10% of the levels in active animals, and return rapidly to near normal levels within 1–2 h as hibernators arouse (Spurrier and Dawe, 1973). Since entrance into hibernation is associated with a rapid and profound leukopenia (greater than 90% drop in levels of circulating leukocytes) it was hypothe-

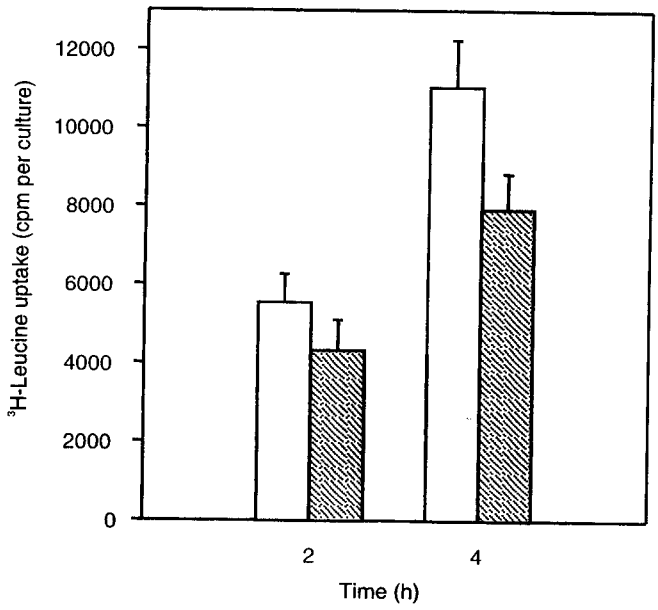

Figure 2. *Inhibition of protein synthesis in 2 or 4 h cultures of EL4 cells by 6% plasma from winter-active versus hibernating 13-lined ground squirrels. Data show mean leucine uptakes of triplicate sets of cultures incubated with plasma from five individual hibernating (hatched bars) or five winter-active (white bars) animals +/– the standard deviation.*

sized that the ischaemic tolerance observed in hibernating animals may, in part, be a consequence of the severely leukocytopenic state. Circulating leukocytes are intimately involved with pathogenic mechanisms associated with ischaemia and reperfusion injury; inflammation-induced adhesion of leukocytes at the blood–endothelial interface proceeds via a coordinated mechanism involving cytokines, chemokines and adhesion molecules. During hibernation, the reduction in circulating leukocytes may be similarly regulated by factors in the blood. Indeed, expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin by rat cerebromicrovascular endothelial cells (EC) were dose-dependently increased by culture with hibernating plasma and to a much lesser extent non-hibernating plasma (Yasuma *et al.*, 1997). Treatment of EC with plasma from hibernating animals similarly induced significantly greater increases in monocyte adhesion to EC (37.2%) than were observed with plasma from active animals (23.9%). Leukocytes and platelets that have become sequestered from the circulation during hibernation do not appear to become further activated and demarginate, but resume circulation as the animals arouse. Therefore, the study of this adaptive response could suggest novel ways to control leukocyte and platelet participation in acute brain ischaemia.

5. Adaptive changes in hibernation

A number of potentially adaptive changes have been observed in the blood of hibernators. Hypocoagulability manifested by prolonged clotting times, a decrease in Factor V concentration (Pivorun and Sinnamon, 1981), reduced Factor VIII and IX, and a 90% drop in circulating platelets (Lechler and Penick, 1963), have been reported. The platelets are sequestered in the red pulp of the spleen (cords of Billroth) and if animals are splenectomized prior to hibernation, thrombocytopenia is prevented and platelets accumulate in liver and lung as small thrombi (Reddick *et al.*, 1973). This suggests that thrombocytopenia in hibernation is a protective mechanism against thrombosis. Reduced blood viscosity (Maclean, 1981) and decreased angiotensin-1-converting enzyme activity (Weekly, 1995) have also been noted during hibernation.

Cerebral cortex slices from hibernators have been shown to have a reduced passive ionic leak of K^+ and a greater Na^+-K^+ pump activity in the cold compared to active animals (that can hibernate) and non-hibernators (Goldman and Willis, 1973). Shchipakina *et al.* (1995) reported phosphorylation of a 53 kDa protein in synaptic membranes isolated from cerebral cortex of hibernating ground squirrels and suggested that protein phosphorylation may be involved in the maintenance of membrane functions during hibernation. Mammals that are capable of entering a hibernating state exhibit a striking tolerance to hypoxia as compared to non-hibernators (Burlington *et al.*, 1969; D'Alecy *et al.*, 1990). Also, hippocampal and septal slices from the brains of hibernating ground squirrels survive in culture far longer than corresponding slices from active ground squirrels or guinea-pigs (Pakhotin *et al.*, 1990).

5.1 *Effect on synaptosomal calcium accumulation*

In addition to regulatory factors in the plasma, other potential mechanisms of cellular adaptation in hibernation may exist. For example, since ion transport requires much of the brain's ATP, it has been proposed that adjustments in membrane conductance and 'ion channel arrest' may occur during anoxia in the brain to decrease the metabolic cost

of maintaining ion gradients and thereby spare energy and promote tolerance to anoxia (Hochachka, 1986). Studies were designed to measure resting and depolarization-induced calcium accumulation in synaptosomes from hibernating as compared to cold-adapted non-hibernating ground squirrels and to identify and characterize the voltage-sensitive calcium channel subtypes present in these subcellular tissue fractions. In these particular experiments, it was hypothesized that during incubation in basal or depolarizing media, calcium accumulation in synaptosomes from hibernating animals would be reduced relative to synaptosomes from non-hibernating animals. Calcium accumulation was measured in synaptosomes that were equilibrated in physiological buffer with 1.2 mM $CaCl_2$ added for 30 min at 37°C. Aliquots were then added to equal volumes of either physiologic buffer with calcium or a depolarizing buffer that contained 50 mM KCl. Each of these buffers contained tracer quantities of $^{45}Ca^{2+}$. Several antagonists were also examined for their ability to inhibit basal and K^+-evoked Ca^{2+} accumulation. The results indicated that there was significantly less $^{45}Ca^{2+}$ accumulation in synaptosomes isolated from hibernating as compared to cold-adapted non-hibernating ground squirrels in both basal ($P<0.005$) and depolarizing ($P<0.03$) media over a 0.5–5 min incubation period (Gentile *et al.*, 1996). The elevation in synaptosomal $^{45}Ca^{2+}$ accumulation triggered by KCl depolarization was blocked by the antagonists ω-conotoxin MVIIC or ω-Agatoxin IVA. These results suggested that the nerve terminals in ground squirrels have calcium channels with a pharmacological sensitivity that shares features of the Q-type calcium channel and indicate that the hibernating state is associated with a decrease in presynaptic $^{45}Ca^{2+}$ conductance via voltage-sensitive channels. The inhibition in calcium accumulation may reflect a cellular adaptation that helps confer tolerance to the profound reduction in cerebral blood flow and capacity for oxygen delivery associated with hibernation.

5.2 *Effect of tyrosine phosphorylation of a brain protein*

Hibernation involves specific cellular regulation rather than being a simple suspension of temperature control. Protein phosphorylation on tyrosine residues, regulated dually by tyrosine kinases and phosphatases, plays an essential role in signal transduction pathways for a wide range of cellular processes. On this basis, it was hypothesized that modulation of protein tyrosine phosphorylation was specific for the hibernating state. Immunoblotting for the phosphotyrosine moiety was used to analyse extracts from various tissues of hibernating and non-hibernating ground squirrels. A single, hibernation-specific phosphoprotein was detected in the brain, but not in any other tissue tested (Ohtsuki *et al.*, 1998). This protein, designated pp98 to reflect its apparent molecular weight, is distributed throughout the brain and is associated with the cellular membrane fraction. The presence of the protein is tightly linked to the hibernation state; it is not present in animals that are exposed to the same cold temperature as the hibernators; it is present for the duration of a hibernation bout (tested from 1–14 days), and disappears within 1 hour of arousal from hibernation. Purification of the protein from 100 brains of hibernating squirrels by preparative isoelectric focusing electrophoresis and subsequent preparative SDS-polyacrylamide gel electrophoresis yielded an estimated 10–50 ng of pp98 based on copper staining of a preparative gel. However, microsequencing by collisionally activated dissociation on a Finnigan TSQ 7000 triple quadrupole mass spectrometer was unsuccessful, as the quantity of isolated protein was still beneath the threshold for

sequence determination. Nonetheless, the close association of pp98 with the hibernation state, its presence in cellular membranes, and the known properties of membrane phosphotyrosine proteins suggest that it may transduce a signal for adaptation to the limited availability of oxygen and glucose and low cellular temperature that characterize hibernation in the ground squirrel.

5.3 *Induction of specific tolerance to hypoxia and aglycaemia*

Tolerance to hypoxia in hibernating tissues was experimentally tested *in vitro* using brain slices from hibernating 13-lined ground squirrels exposed to hypoxia and aglycaemia. Hippocampal slices from ground squirrels in both the active and hibernating states and from rats were subjected to *in vitro* hypoxia and aglycaemia at incubation temperatures of 36°C, 20°C and 7°C and evaluated histologically. A binary bioassay (intact vs. severely damaged CA1 hippocampal neurons) was used to determine the duration of hypoxia/aglycaemia tolerated in each group. At 36°C, hippocampal neurons from active ground squirrels showed a necrotic response (poorly staining, ballooned neurons) after 5 min exposure to hypoxia, whereas the hibernating tissue did not show a necrotic response until 8 min of exposure (Frerichs and Hallenbeck,1998a). Furthermore, this tolerance to hypoxia *in vitro* was rapidly induced, since it was observed in animals after only 4 h of hibernation. At all temperatures, slices from hibernating animals were the most tolerant compared with active squirrels or with rats; hippocampal slices from hibernating animals maintained normal morphology for longer intervals during hypoxia and aglycaemia than identically treated hippocampal slices from non-hibernating ground squirrels. These results indicate that hibernation is not only a state in which homeostasis is preserved during severely reduced blood flow and limited oxygen availability, but that it is additionally a state of tolerance to superimposed hypoxic and aglycaemic stress that disrupts homeostasis. Studies indicate that these hibernating tissues may have a reduced metabolic rate, which is attributed to reversible modifications in several enzymes of glucose metabolism.

6. Suppression of protein synthesis in brain during hibernation

Protein synthesis *in vivo* in hibernating 13-lined ground squirrels was found to be below the limit of L-[1-^{14}C]leucine autoradiographic detection. In brain extracts of hibernating squirrels, protein synthesis was determined to be 0.04% of the average rate of active squirrels (Frerichs *et al.*, 1998b). Furthermore, synthesis was reduced three-fold in cell-free extracts from hibernating brain (incubated at 37°C); this eliminates hypothermia as the only cause for the inhibition of protein synthesis. In these studies, suppression of protein synthesis was shown to involve blocks on both initiation and elongation, and its onset coincided with the early transition phase into hibernation. An increased monosome peak with moderate ribosomal disaggregation in polysome profiles and greatly increased phosphorylation of eIF2α (immunoblotting) were both consistent with an initiation block in hibernators. The elongation block was demonstrated by a three-fold increase in ribosomal mean transit times in cell-free extracts from hibernators. No abnormalities of ribosomal function or mRNA levels were detected. These surprising findings implicate suppression of protein synthesis as a component of the regulated shutdown of cellular function that permits hibernating ground squirrels to tolerate greatly reduced blood flow, substrate

and oxygen availability. Further study of the factors that control protein synthesis may lead to identification of the molecular mechanisms that regulate hibernation.

7. Changes in gene expression during hibernation

A number of changes in gene expression have been reported in hibernation. Kondo and Kondo (1992) identified four 'hibernation' proteins in chipmunk liver that decreased during the hibernation season and demonstrated that their mRNAs showed homology to the α1-antitrypsin gene family (Takamatsu *et al.*, 1993, 1997). Both the mRNA and protein levels of a broad-spectrum protease inhibitor and inhibitor of Factor Xa, α_2-macroglobulin, were found to undergo a state-specific increase in the liver of hibernating as compared to active ground squirrels (Srere *et al.*, 1992, 1995). Genes for pancreatic lipase (liberates fatty acids from triglycerides) and pyruvate dehydrogenase kinase isozyme 4 (depresses metabolism by preventing conversion of pyruvate to acetyl-CoA) are upregulated in ground squirrel heart during hibernation (Andrews *et al.*, 1998). The observations by Andrews *et al.* (1998) provide evidence that differential expression of genes contribute to adaptive changes in cardiac physiology during hibernation because fatty acids are made available for metabolism and metabolic rate is reduced.

7.1 *Melatonin receptor gene expression*

As discussed above, hibernation in mammals is widely recognized as a seasonal adaptation for energy conservation. Melatonin, secreted by the pineal body, is linked with sending seasonal (calendar) as well as daily (clock) signals to the organism (Reiter, 1993) and has also been linked to the regulation of core body temperature in mammals (Saarela and Reiter, 1994; Cagnacci *et al.*, 1997). Melatonin also intervenes in generating seasonal rhythms of daily torpor and hibernation and in heat stress tolerance. Administration of melatonin could alter the thermogenic capacity and make thermoregulatory adjustments necessary for hibernation (Viswanathan *et al.*, 1986); it may also prolong the length of hibernation bouts of ground squirrels (Stanton *et al.*, 1987). Recent reports indicate that melatonin also serves as an endogenous antioxidant agent with a proficient free radical scavenging activity (Reiter *et al.*, 1994, 1999a, 1999b) and may also play a protective role in toxic chemical-induced neurotoxicity in the rat hippocampus (Tan *et al.*, 1998). Furthermore, this pineal hormone can induce sleep in humans; this hypnotic effect may be separated from its effects on circadian rhythms (Dollins *et al.*, 1994). Evidence from electroencephalographic, thermoregulatory and cellular neurophysiological studies suggest that sleep and hibernation may be homologous adaptations for energy conservation (Kilduff *et al.*, 1993). These observations indicate that melatonin may play a key role in induction and maintenance of hibernation.

The effects of melatonin are mediated by pharmacologically specific, high-affinity melatonin receptors that have been identified and characterized in a number of tissues by *in-vitro* autoradiography and conventional binding assays using [^{125}I] iodomelatonin as the ligand (Dubocovich and Takahashi, 1987; Morgan *et al.*, 1994; Dubocovich, 1995). The cDNA of the melatonin receptor was cloned from *Xenopus* dermal melanophores in 1994 (Ebisawa *et al.*, 1994). Based on structural analysis, the melatonin receptor belongs to a distinct group within the superfamily of G protein-coupled

receptors. Presently, three subtypes of the melatonin receptor have been found: Mel_{1A} expressed in mammalian and bird brain, Mel_{1B} expressed mainly in mammalian retina, and Mel_{1C} detected in amphibian melanophores, brain and retina; and in bird and fish brains (Reppert *et al.*, 1995, 1996). Melatonin receptors were also found in other organs and tissues, such as heart, liver, intestine, kidney, lung, spleen, brown adipose tissue and blood vessels (Pang *et al.*, 1993a,b, 1996; Viswanathan *et al.*, 1993; Acuna-Castroviejo *et al.*, 1994; Capsoni *et al.*, 1994; Le Gouic *et al.*, 1997; Drew *et al.*, 1998). The wide distribution of melatonin receptors suggests that their ligand, melatonin, has an essential physiological function in humans and animals. The melatonin receptor densities and their regulation in the hypothalamic suprachiasmatic nuclei (SCN) and in the hypophysial pars tuberalis (PT) of rats and other rodents have been investigated (Gauer *et al.*, 1994; Masson-Pevet and Gauer, 1994). Since Mel_{1A} forms the majority of melatonin receptors in mammalian brain including SCN and PT, it is presumed that this subtype may mediate seasonal and circadian effects of melatonin. This hypothesis is further supported by the natural knockout of Mel_{1B} receptor in Siberian hamsters (Weaver *et al.*, 1996) and by the detection (*in situ* hybridization) of only Mel_{1A}, but not Mel_{1B} receptor mRNA in human SCN (Weaver and Reppert, 1996). However, expression of the melatonin receptor gene has not been extensively studied in the brains of hibernating versus non-hibernating animals.

Since heart is a contractile organ that must continue to work despite hypothermia and low oxygen supply, ground squirrels exhibiting seasonal changes in their energy expenditure would benefit if their cardiac system responded to melatonin. Melatonin receptors have been observed in hearts of quail and duck (Pang *et al.*, 1993b, 1996) suggesting that melatonin has an effect on the cardiovascular function of birds. In hibernating animals, tremendous changes in body temperature and heart rate must be controlled and regulated by the differential expression of certain genes closely related to conserving energy and reducing metabolism. Also, as previously mentioned, the gene expressions of pancreatic lipase and pyruvate dehydrogenase kinase isozyme 4 were shown to be up-regulated in the heart of hibernating 13-lined ground squirrel in order to inhibit carbohydrate metabolism (Andrews *et al.*, 1998). Melatonin receptor gene expression during hibernation may be similarly involved in this metabolism-reducing effect.

Brown adipose tissue (BAT) is a highly specialized type of adipose tissue found in newborn mammals and some hibernating animals. BAT has a relatively large cytoplasm, which is strongly stained due to a large content of mitochondria. Thyroxine 5′-deionation in BAT of Richardson's ground squirrels is greatly elevated following melatonin treatment and this suggests that melatonin is involved in the thermogenic regulation (Puig-Domingo *et al.*, 1988). Recently, it was reported that daily melatonin administration to middle-aged male rats suppresses body weight, intra-abdominal adiposity and plasma leptin and insulin (Wolden-Hanson *et al.*, 2000). This denotes that melatonin has an energy-saving effect in rodents. Since leptin is synthesized in and secreted from white and brown adipose tissue and appears to fulfil many properties of a satiety factor, to decrease food intake and increase energy expenditure (Campfield *et al.*, 1995), the decreases of serum leptin and insulin that are stimulated by melatonin suggest that the hormone causes a suppression of energy expenditure and glucose metabolism. Furthermore, the effect of melatonin on inhibition of lipolysis and lipogenesis has also been shown in isolated adipocytes (Ng and Wong, 1986). Interestingly, melatonin binding sites have also been detected in Siberian hamster BAT (Le Gouic *et al.*, 1997).

The findings presented here evaluate the mel_{1a} gene expression in telencephalon, heart and brown adipose tissue of hibernating and non-hibernating (warm active and cold exposed) 13-lined ground squirrels (*Spermophilus tridecemlineatus*). Expression of mel_{1a} in hibernating ($n = 5$), cold-adapted active ($n = 5$) and warm active 13-lined ground squirrels ($n = 5$) was determined by *in situ* hybridization with a biotinylated rat oligonucleotide probe (GAGAGTTCCG GTTTGCAGGT TGGGCATGAT GGCT, GenBank Accession number U14407). The *in situ* hybridization technique was similar to the method previously reported (Rollwagen *et al.*, 1998). Sections were deparaffinized and permeabilized with proteinase K (2.5 $\mu g\ ml^{-1}$) for 10 min at 37°C followed by acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature. Incubation with a biotinylated oligo probe was performed overnight at 37°C; this probe was designed by us and chemically synthesized and biotinylated at the 5′ end by GenSet Corp. (La Jolla, CA). Posthybridization washes (four times in 1 × stringent wash solution (DAKO Corp., Carpinteria, CA)) were undertaken at 15°C below the melting temperature (T_m) of the probe. After blocking of nonspecific binding sites with blocking buffer (3% BSA, 0.3% Tween 20, in 50 mM Tris buffer (pH 7.4)/200 mM NaCl)), the signals were detected histochemically by subsequent incubation with streptavidin-alkaline phosphatase (AP) conjugate (DAKO Corp.). The purple blue colour reaction was developed in bromochloroindolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) substrate.

The results (*Table 2*) demonstrated that warm-active animals exhibited low levels of mel_{1a} mRNA in brain, heart and BAT. All hibernating animals expressed significantly elevated levels of mel_{1a} mRNA in brain, especially in the cortex, hippocampus (particularly in CA1), and hypothalamus (especially in supraoptic nucleus, and preoptic nuclei) (*Figure* 3) as well as in cardiac muscle and BAT (*Figure* 4). Intermediate levels of mel_{1a} gene

Table 2. Mel_{1a} *mRNA expression*[a]

Tissue	Hibernating	Cold-adapted	Warm-active
CA1 area of hippocampus	0.408 ± 0.026	0.376 ± 0.035	0.263 ± 0.033
Forebrain cortex	0.438 ± 0.077	0.346 ± 0.050	0.233 ± 0.031
Hypothalamus	0.507 ± 0.049	0.314 ± 0.064	0.186 ± 0.037
Myocardium	0.383 ± 0.036	0.203 ± 0.029	0.098 ± 0.021
Brown adipose tissue	0.596 ± 0.039	0.269 ± 0.041	0.138 ± 0.036

[a]After *in situ* hybridization, tissue images were acquired using an Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD) through a colour video camera (Toshiba, 3CCCD). Slides were viewed using a 40× objective on an Olympus AH3 light microscope. Image processing was performed for identification and density level of the purple blue (BCIP/NBT) signals for the presence and quantity of Mel_{1a} transcripts in indicated areas. The intensity of alkaline phophatase histostaining (after hybridization) in each brain section was calibrated from a background (white) level set at 0, and a value depicting the most intense (dense) level of staining (black) set at 1. Four slides of brain, heart or BAT were examined for each animal in the hibernating ($n = 6$), cold-adapted ($n = 5$), and warm-active ($n = 6$) groups. Eight to ten fields of each anatomical region on each slide were analysed. The mean optical density (mean ± SD) of all fields of each tissue group was calculated and exported as a text file. The text file was imported into the MicroSoft Excel program and statistical analysis was performed on an IBM PC-type (Pentium II) computer using SigmaPlot and SigmaStat software (Jandel scientific, San Rafael, CA). The data from each group of animals (presented as mean ± SD) were compared using one-way ANOVA by SigmaStat program. For each tissue shown, all values were significantly ($P < 0.05$) different between each of the three groups.

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ted	Warm-active
035	0.263 ± 0.033
050	0.233 ± 0.031
064	0.186 ± 0.037
029	0.098 ± 0.021
041	0.138 ± 0.036

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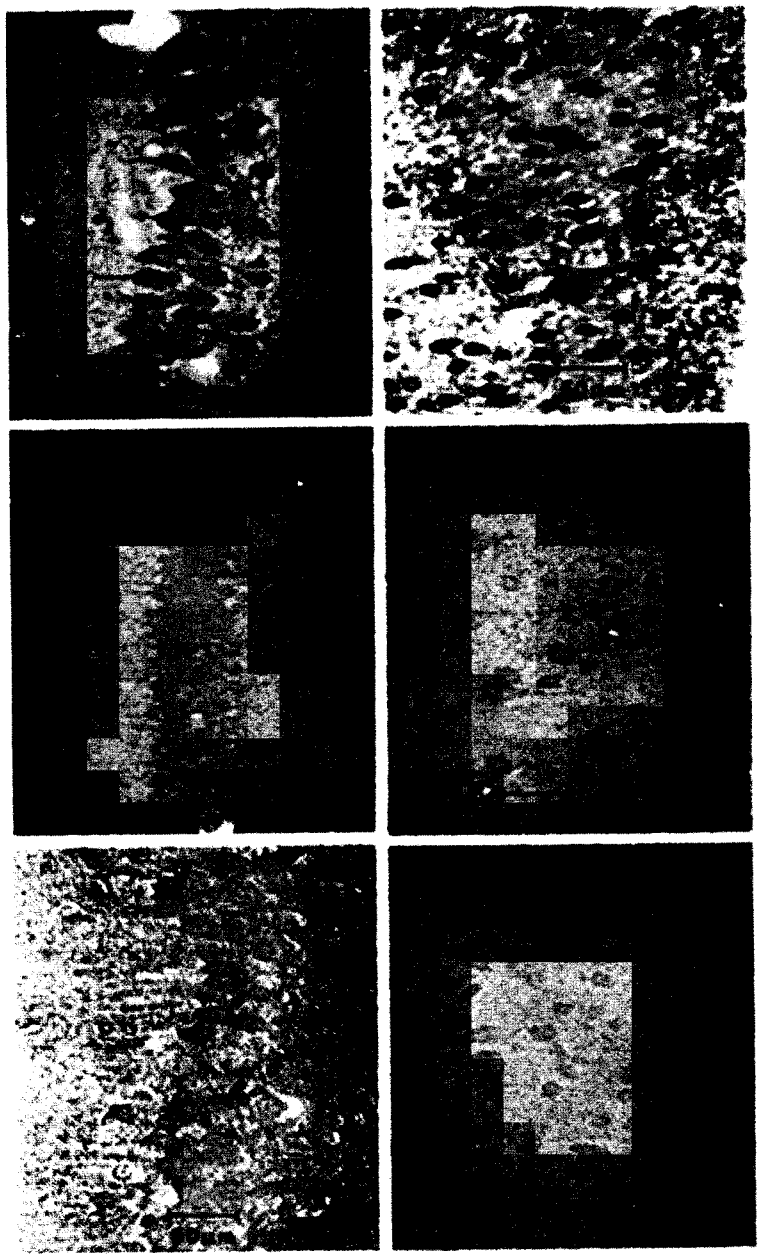


Figure 3. *The differential expression of melatonin receptor* Mel_{1a} *mRNA in the brain of hibernating (top row), cold exposed (middle row) and warm active 13-lined ground squirrels (bottom row). In each row, the right-hand photo is* in situ *hybridization for melatonin receptor* (Mel_{1a}) *mRNA in the cerebral cortex, and the left-hand photo is* Mel_{1a} *mRNA in the CA1 region of hippocampus. The range of staining intensities (detected by streptavidin-AP system, blue purple colour) reflects the specific* Mel_{1a} *mRNA levels. A significantly higher level of* Mel_{1a} *mRNA in brain is seen in the hibernating group (top) and the lowest level is in warm active animals (bottom). The* Mel_{1a} *mRNA expression level in cold exposed squirrels is intermediate between the above two groups.*

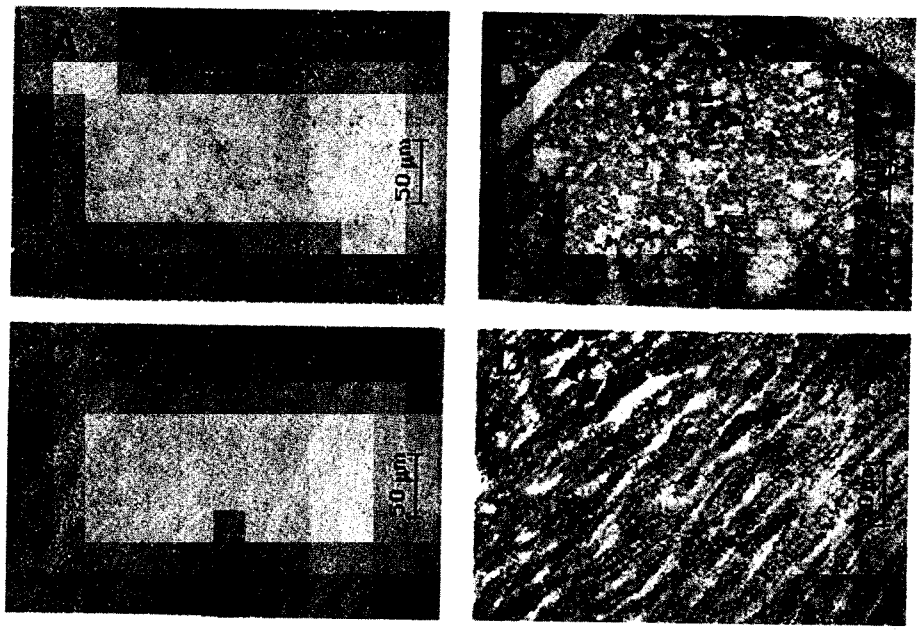


Figure 4. The differential expression of melatonin receptor Mel_{1a} *mRNA in the heart and brown adipose tissue of hibernating and warm active 13-lined ground squirrels. The photos are* in situ *hybridization for melatonin receptor* (Mel_{1a}) *mRNA expression in BAT (A, B) and heart (C, D) from active (A, C) and hibernating (B, D) animals.*

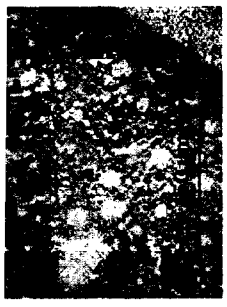
expression were found in the cold-adapted animals. These data suggest that melatonin may be linked to energy-saving, metabolism-reducing and/or thermoregulatory mechanisms associated with induction and maintenance of hibernation in 13-lined ground squirrels.

8. Summary

Hibernation is examined in light of its many physiological adaptations that may lead to beneficial approaches to treatment of stroke, haemorrhagic shock and organ ischaemia. These medical problems are sufficiently complex and involve a plethora of interactions resulting in a maze of pathophysiological responses. Hibernation may prove to be a guide through this maze allowing us to understand the regulation of physiological responses associated with induction of tolerance to hypoxia. This area of research, which investigates the creation of low metabolic states, may ultimately identify mechanisms to promote cell survival during haemorrhagic shock and organ ischaemia.

Acknowledgements

This work was supported, in part, by the Office of Naval Research, Work Unit 602233N-03330-120-A0034.



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